Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10535–10539, December 1991
Medical Sciences

Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin

(septic shock/cachectin/lymphotoxin/immunoglobulin chimera)

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Communicated by David R. Davies, August 19, 1991 (received for review June 13, 1991)

ABSTRACT **Tumor necrosis factors (TNF) α and β are structurally related cytokines that mediate a wide range of immunological, inflammatory, and cytotoxic effects. During bacterial infection of the bloodstream (sepsis), TNF-α induction by bacterial endotoxin is thought to be a major factor contributing to the cardiovascular collapse and critical organ failure that can develop. Despite antibiotic therapy, these consequences of sepsis continue to have a high mortality rate in humans. Here we describe a potent TNF antagonist, a TNF receptor (TNFR) immunoadhesin, constructed by gene fusion of the extracellular portion of human type 1 TNFR with the constant domains of human IgG heavy chain (TNFR-IgG). When expressed in transfected human cells, TNFR-IgG is secreted as a disulfide-bonded homodimer. Purified TNFR-IgG binds to both TNF-α and TNF-β and exhibits 6- to 8-fold higher affinity for TNF-α than cell surface or soluble TNF receptors. *In vitro*, TNFR-IgG blocks completely the cytolytic effect of TNF-α or TNF-β on actinomycin D-treated cells and is markedly more efficient than soluble TNFR (24-fold) or monoclonal anti-TNF-α antibodies (4-fold) in inhibiting TNF-α. *In vitro*, TNFR-IgG prevents endotoxin-induced lethality in mice when given 0.5 hr prior to endotoxin and provides significant protection when given up to 1 hr after endotoxin challenge. These results confirm the importance of TNF-α in the pathogenesis of septic shock and suggest a clinical potential for TNFR-IgG as a preventive and therapeutic treatment in sepsis.**

Tumor necrosis factors α (TNF-α; cachectin) and β (TNF-β; lymphotoxin) are related proteins, secreted by activated macrophages and lymphocytes, respectively (1–3). These cytokines have been implicated in diverse biological processes including immunoregulation, inflammation, antiviral defense, cachexia, angiogenesis, and septic shock. The biological effects of TNF-α and TNF-β are mediated through specific receptors. Molecular cloning has demonstrated the existence of two distinct types of TNF receptor (TNFR), each of which binds to both TNF-α and TNF-β (4–8). The extracellular portions of both receptors are found naturally also as soluble TNF binding proteins (7, 8).

Several lines of evidence indicate that TNF-α is a principal mediator in the pathogenesis of septic shock. First, neutralizing anti-TNF-α antibodies can prevent the pulmonary failure and death associated with administration of endotoxin or *Escherichia coli* in mice (9) or baboons (10). Second, intravenous infusion of TNF-α leads to a toxic syndrome indistinguishable from that caused by endotoxemia and gram-negative sepsis (11, 12). In addition, the levels of TNF-α increase substantially in the circulation of animals and humans who have received endotoxin or have septic shock (13, 14) and correlate with mortality in severe sepsis (15–17).

To create a TNF antagonist that might block the lethal effect of TNF in endotoxic shock, we constructed an immunoadhesin (18) containing the extracellular portion of human type 1 TNFR and the hinge and Fc regions of human IgG heavy chain (TNFR-IgG). This approach was based on the observation that the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), can be linked to IgG heavy chain, thus creating a protein with two functional HIV binding sites and a markedly longer plasma half life than the soluble extracellular portion of CD4 (18, 19). We show that the TNFR-IgG chimera acts as a potent antagonist of TNF-α and TNF-β *in vitro* and can prevent endotoxin-induced lethality in a mouse model for septic shock. These results suggest that TNFR-IgG may be useful against the potentially lethal consequences of sepsis in humans.

MATERIALS AND METHODS

Construction, Expression, and Purification of TNFR-IgG. The schematic structure of TNFR-IgG is shown in Fig. 1*A*. A mammalian expression vector encoding TNFR-IgG (pRK-TNFR1-IgG) was constructed from plasmids encoding the human type 1 TNFR (pRK-TNF-R) (4) and CD4-IgG ($pRKCD4_2Fc_1$) (19). A 770-base-pair (bp) DNA fragment containing 5′ untranslated sequences and encoding the leader and extracellular portion of type 1 TNFR was generated by digesting pRK-TNF-R with *Eco*RI and *Hin*dIII. Plasmid $pRKCD4_2Fc_1$, encoding the extracellular domain of CD4 fused to the hinge and Fc region of human IgG_1 heavy chain (19), was digested with *Eco*RI and *Nde* I to remove most of the CD4 sequence while retaining the IgG_1 sequence. The TNFR-encoding fragment was then inserted 5′ of the IgG_1 sequence and in the same reading orientation by ligating the respective *Eco*RI sites and by blunting and ligating the *Hin*dIII and *Nde* I sites. The remaining CD4 sequence was removed to create the exact junction between threonine-171 of TNFR and aspartic acid-216 of IgG_1 heavy chain by oligonucleotide-directed deletional mutagenesis, using synthetic oligonucleotides complementary to the 24 nucleotides at the borders of the desired TNFR and IgG_1 fusion sites as primers and the plasmid described above as a template (18). The final DNA construct was sequenced to confirm the correct primary structure. The mature TNFR-IgG polypeptide encoded by pRKTNFR-IgG thus contains 171 residues from TNFR and 227 residues from IgG_1—i.e., a total of 398

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Abbreviations: TNF, tumor necrosis factor; TNFR, TNF receptor; C_H, heavy-chain constant region; V_H, heavy-chain variable region; TNFR-IgG, fusion of TNFR with IgG_1 heavy-chain hinge region and C_H2 and C_H3 domains; HIV, human immunodeficiency virus; sTNFR, soluble TNFR.

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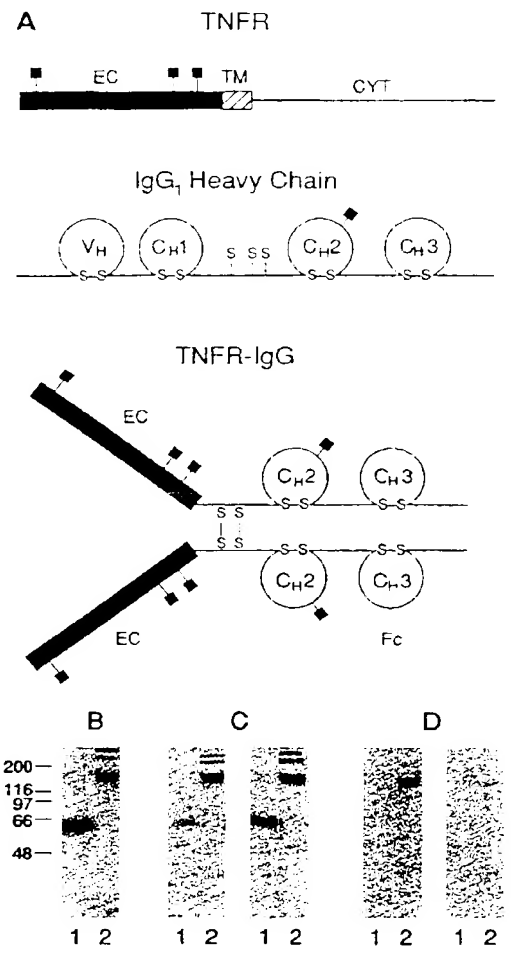

FIG. 1. (*A*) Schematic structure of the human type 1 TNFR, human IgG_1 heavy chain, and TNFR-IgG. The extracellular (EC), transmembrane (TM), and cytoplasmic (CYT) domains of TNFR and the IgG_1 heavy-chain variable region (V_H) and constant region (domains C_H1, C_H2, and C_H3) are indicated. TNFR-IgG was constructed by gene fusion of the extracellular domain of TNFR with the hinge region and C_H2 and C_H3 domains of the IgG_1 heavy chain. Potential asparagine-linked glycosylation sites (■) and disulfide bonds (S-S) (IgG protein only) are shown also. (*B–D*) Subunit structure and functional domains of TNFR-IgG. Human embryonic kidney 293 cells were transfected with a vector directing transient expression of TNFR-IgG. The protein was recovered from culture supernatants and purified by affinity chromatography on *S. aureus* protein A. SDS/polyacrylamide gel electrophoresis was carried out under reducing (lane 1) or nonreducing (lane 2) conditions. The proteins were stained with Coomassie blue (B) or electroblotted onto nitrocellulose paper and incubated with antibodies to human TNFR (*C Left*) or human IgG Fc (*C Right*) or with ^{125}I-TNF-α (1 nM) in the absence (*D Left*) or presence (*D Right*) of unlabeled TNF-α (100 nM). Blots were developed with horseradish peroxidase-conjugated second antibody (*C*) or autoradiography (*D*).

amino acids. TNFR-IgG was expressed in human embryonic kidney 293 cells by transient transfection with pRKTNFR-IgG by the calcium phosphate precipitation method as described (18). TNFR-IgG was purified to >95% homogeneity from serum-free cell culture supernatants by affinity chromatography on *Staphylococcus aureus* protein A. TNFR-IgG was eluted with 50 mM sodium citrate, pH 3/20% (wt/vol) glycerol, and the pH was neutralized with 0.05 vol of 3 M Tris·HCl (pH 8–9).

TNF Binding Assays. Binding of TNFR-IgG to TNF was analyzed essentially as described for CD4-IgG binding to HIV gp120 (20). TNFR-IgG (1 μg/ml) was immobilized onto microtiter wells coated with goat anti-human IgG Fc antibody. Reactions with recombinant human ^{125}I-labeled TNF-α (^{125}I-TNF-α; radioiodinated by using lactoperoxidase to a specific activity of 19.1 μCi/μg; 1 μCi = 37 kBq) were done in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 1 hr at 24°C. Nonspecific binding was determined by omitting TNFR-IgG. In competition binding analyses, ^{125}I-labeled TNF-α was incubated with immobilized TNFR-IgG in the presence of increasing concentrations of unlabeled TNF. The K_d was determined from competition IC_{50} values according to the following equation: $K_d = IC_{50}/(1 + [T]/K_{dT})$, where [T] is the concentration of the tracer (0.1 nM) and K_{dT} is the K_d of the tracer determined by saturation binding (80 pM).

TNF Cytotoxicity Assays. TNF cytotoxicity was assayed essentially as described (21). Murine L-M cells were plated in microtiter dishes (4×10^4 cells per well) and treated with actinomycin D (3 μg/ml) and TNF-α or TNF-β (1 ng/ml) in the absence or presence of TNFR-IgG or other inhibitors. After 20 hr of incubation at 39°C, the cell survival was determined by a crystal violet dye exclusion test.

Mouse Model for Septic Shock. Septic shock was modeled by endotoxin injection of 6- to 8-week-old female BALB/c mice. Animals were injected intravenously (i.v.) with an LD_{100} dose of *Salmonella abortus*-derived endotoxin (175 μg per mouse) in phosphate-buffered saline (PBS), and survival was followed for at least 78 hr. Purified TNFR-IgG, or CD4-IgG used as a negative control, were diluted in PBS and injected i.v. prior to or after endotoxin administration.

RESULTS

Subunit Structure of TNFR-IgG. TNFR-IgG was created by fusing complementary DNAs encoding the extracellular portion (amino acids 1–171) of human 55-kDa type 1 TNFR and the hinge region and constant region C_H2 and C_H3 domains (amino acids 216–443) of human IgG_1 heavy chain (Fig. 1*A*). A vector directing mammalian expression of TNFR-IgG was introduced transiently into human kidney 293 cells to produce the molecule as a secreted protein. Taking advantage of the presence of an IgG Fc domain in TNFR-IgG, we used protein A affinity chromatography to recover and purify the protein from cell culture supernatants. We examined the subunit structure of TNFR-IgG by SDS/polyacrylamide gel electrophoresis (Fig. 1 *B–D*). Under reducing conditions, a molecular mass of ≈60 kDa was observed, whereas under nonreducing conditions, it was approximately doubled, indicating that TNFR-IgG is a disulfide-bonded dimer (Fig. 1*B*). Minor bands of higher molecular mass were observed also, suggesting some aggregation of TNFR-IgG. Western blot analyses showed reactivity of TNFR-IgG with antibody to the type 1 human TNFR or to human IgG Fc (Fig. 1*C*) and showed specific binding of human ^{125}I-TNF-α (Fig. 1*D*). Notably, ^{125}I-TNF-α did not bind to reduced TNFR-IgG, suggesting that intramolecular disulfide bonds in TNFR are required for binding to TNF-α. These results indicate a covalent homodimeric structure for TNFR-IgG and the presence of functional TNF binding and antibody Fc domains in this protein.

Binding of TNFR-IgG to TNF-α and TNF-β. To investigate the binding of TNFR-IgG to TNF-α and TNF-β, we carried out saturation and competition binding analyses, using an assay in which TNFR-IgG was immobilized by binding of its Fc domain to anti-Fc antibodies coated on microtiter wells. Human ^{125}I-TNF-α bound to a single class of sites with an apparent dissociation constant (K_d) of 80 ± 20 pM (Fig. 2 *Left*). Human TNF-β was able to displace the binding of ^{125}I-TNF-α completely, confirming previous observations

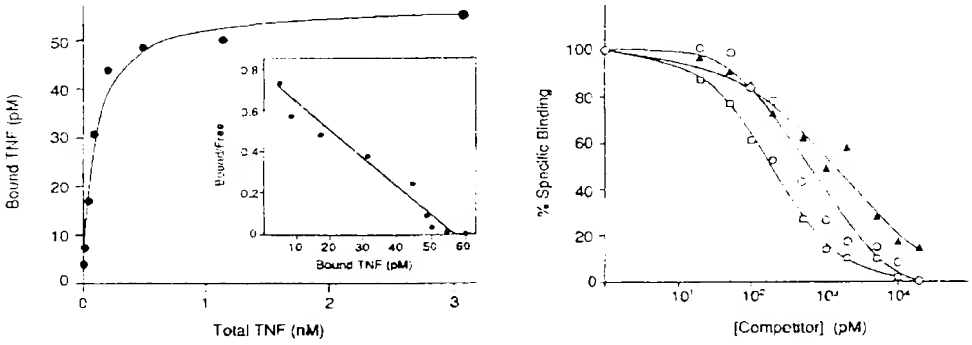

FIG. 2. Binding of TNFR-IgG to TNF-α and TNF-β. (*Left*) Saturation analysis of TNFR-IgG binding to human TNF-α. Purified TNFR-IgG was immobilized in microtiter wells coated with anti-IgG Fc antibody and incubated with increasing concentrations of recombinant human ^{125}I-TNF-α. Saturation and Scatchard (*Inset*) plots were generated by using the best fit as determined by unweighted least-squares regression analyses. (*Right*) Competition analysis of TNFR-IgG binding to recombinant human TNF-α (○), human TNF-β (▲), and murine TNF-α (□). TNFR-IgG was incubated with 100 pM ^{125}I-TNF-α in the presence of increasing concentrations of unlabeled competitor.

that both TNFs bind to type 1 TNFR (4–8). A K_d of 550 ± 100 pM was observed for TNF-β and a K_d of 75 ± 5 pM was observed for murine TNF-α (Fig. 2 *Right*). Notably, the K_d for binding of TNFR-IgG to TNF-α was significantly lower than values reported for type 1 cell-surface or soluble TNFR (sTNFR) (470–660 nM) (4–6). Thus, TNFR-IgG appears to bind to TNF-α with 6- to 8-fold higher affinity than type 1 cell surface TNFR or sTNFR. This higher affinity may be due to a multivalent interaction between TNFR-IgG and TNF-α, as the structure of TNFR-IgG is dimeric (Fig. 1) and the structure of TNF-α is trimeric (22–24). Indeed, saturation analysis in solution, in which complexes of ^{125}I-TNF-α and TNFR-IgG were precipitated quantitatively with protein A, showed a molar binding ratio of trimeric TNF-α and TNFR-IgG of 1.25 ± 0.05:1 (not shown). These results are consistent with the possibility that the two TNFR domains of a TNFR-IgG molecule interact with one TNF-α trimer, which may result in a more stable binding interaction. Alternatively, only one of two TNFR domains in TNFR-IgG may interact with a TNF-α trimer; this is less likely, however, since it would not be expected to result in higher binding affinity.

TNFR-IgG Blocks the Cytolytic Actions of TNF-α and TNF-β *in Vitro*. To test the ability of TNFR-IgG to antagonize TNF activity *in vitro*, we investigated the effect of TNFR-IgG on the induction of cell lysis by TNF-α in actinomycin D-treated murine L-M cells (Fig. 3 *Left*). While no inhibitory effect was observed with CD4-IgG, TNFR-IgG was able to block cell killing completely, with 50% inhibition (IC_{50}) occurring at 0.5 μg/ml (5 nM). For comparison, we tested type 1 sTNFR and found an IC_{50} of 3.5 μg/ml (120 nM). In addition, we tested the activity of two highly neutralizing monoclonal antibodies to human TNF-α (25) and found an IC_{50} of ≈3.5 μg/ml (≈21 nM). Thus, on a molar basis, TNFR-IgG was 24-fold more efficient than sTNFR and 4.2-fold more efficient than anti-TNF-α antibodies in blocking the cytolytic action of TNF-α. We tested also the ability of TNFR-IgG to block the cytolytic activity of TNF-β (Fig. 3 *Right*). Complete inhibition of cell killing was achievable, with an IC_{50} of 1.5 μg/ml (15 nM). Thus, TNFR-IgG was less efficient by a factor of 3 in blocking TNF-β than TNF-α, consistent with its lower affinity for TNF-β. These results show that TNFR-IgG acts as a full antagonist *in vitro* against both types of TNF.

TNFR-IgG Protects Against Septic Shock in Mice. To investigate the ability of TNFR-IgG to act as a TNF antagonist *in vivo*, we used a model for septic shock in mice (Fig. 4). In animals receiving an LD_{100} dose of endotoxin, complete lethality was observed within 48 hr. Injection of TNFR-IgG 0.5 hr prior to endotoxin administration prevented lethality at a TNFR-IgG dose of 20 μg per mouse and provided partial protection at lower doses, whereas CD4-IgG had no significant effect (Fig. 4 *Left*). We investigated the temporal relation of TNFR-IgG and endotoxin injection also (Fig. 4 *Right*). Injection of 10 μg of TNFR-IgG per mouse provided signif-

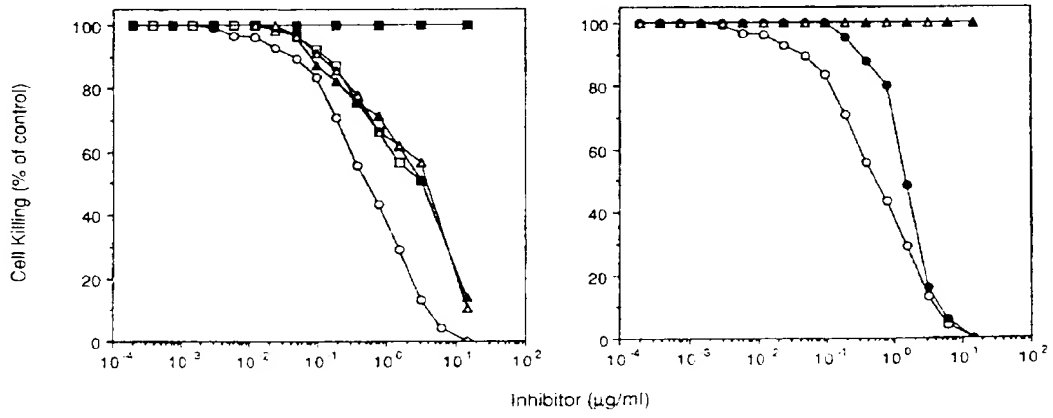

FIG. 3. Inhibition of TNF cytotoxicity by TNFR-IgG *in vitro*. (*Left*) Effect of TNFR-IgG (○), soluble type 1 TNFR (□), monoclonal antibodies D (△) or E (▲) to human TNF-α (15), or CD4-IgG (■) (11) on the killing of actinomycin D-treated murine L-M cells induced by TNF-α (1 ng/ml). (*Right*) Effect of TNFR-IgG (○, ●) or CD4-IgG (△, ▲) on cell killing by TNF-α (1 ng/ml) (○, △) or TNF-β (1 ng/ml) (●, ▲).

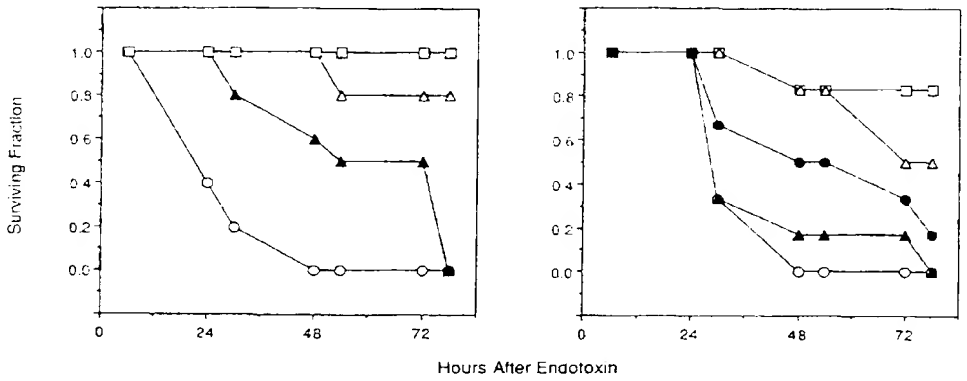

FIG. 4. Inhibition of endotoxin-induced lethality in mice by TNFR-IgG. (*Left*) Effect of the dose of TNFR-IgG on endotoxin-induced lethality. Mice (n = 10) were given an i.v. injection of TNFR-IgG at a dose per mouse of 20 μg (□), 4 μg (△), and 0.8 μg (▲) or CD4-IgG at 4 μg per mouse (○). Thirty minutes later, the mice were given an i.v. injection of endotoxin. Survival data is shown for the first 78 hr and remained unchanged for at least another week. (*Right*) Effect of the time of administration of TNFR-IgG in relation to the time of endotoxin challenge. Mice (n = 6) were given i.v. injection of endotoxin only (○) or of TNFR-IgG (10 μg per mouse) 0.5 hr before (□), 0.5 hr after (△), 1 hr after (●), or 2 hr after (▲) administration of endotoxin.

icant protection 0.5 hr before, 0.5 hr after, or 1 hr after endotoxin injection but little protection 2 hr after endotoxin injection. These data show that TNFR-IgG can prevent or significantly delay endotoxin-induced lethality in mice when given prior to or shortly after endotoxin challenge.

DISCUSSION

Our results show that TNFR-IgG, a molecule that combines the TNF binding function of the extracellular portion of type 1 TNFR with the dimeric structure of IgG, is a potent TNF antagonist. At the molecular level, TNFR-IgG exhibits significantly higher affinity for TNF-α than monomeric cell surface or soluble TNFR, and a molar binding ratio of 1.25:1 TNF-α trimer to TNFR-IgG suggests that this higher affinity may be due to bivalent binding to TNF-α.

At the cellular level, TNFR-IgG blocks the cytolytic action of TNF-α or TNF-β in murine L-M cells completely, and is markedly more potent than sTNFR or anti-TNF-α monoclonal antibodies in blocking TNF-α. The difference in TNF-α binding affinity between TNFR-IgG and sTNFR probably contributes to the differential efficiency of these forms of TNFR in blocking the TNF-α cytolytic activity. However, the difference between TNFR-IgG and sTNFR in blocking TNF-α (24-fold) is significantly greater than the difference in affinity (6- to 8-fold). Previous work with anti-TNFR antibodies showed that bivalent but not monovalent antibody fragments can activate TNFR (26), indicating that a TNF-α trimer may trigger signal transduction by cross-linking two cell surface TNFR molecules. Therefore, the ability of TNFR-IgG to block two receptor binding sites on a TNF-α trimer simultaneously, thus rendering TNF-α unable to dimerize cell surface receptors, also may contribute to the greater efficiency of TNFR-IgG vs. sTNFR in blocking TNF-α.

At the level of the whole organism, TNFR-IgG can prevent or protect against endotoxic shock in mice, depending on the dose and time of injection. This confirms the hypothesis that TNF-α is a key contributor to the septic shock syndrome, first suggested by the ability of anti-TNF-α antibodies to protect against septic shock (9, 10). The ability of TNFR-IgG to provide protection at the doses tested in this study appears limited to about 1 hr after endotoxin challenge. This is consistent with the finding that the rise in circulating levels of TNF-α in animals challenged with endotoxin or *E. coli* and in patients with septic shock is transient (13–17). Taken together, these observations support the notion that the transient increase in TNF-α following sepsis triggers a subsequent cascade of events that can lead to the pathogenesis of shock and multiple organ failure.

In the past few decades, major advances in the treatment of bacterial infections have been achieved, such as the development of powerful antimicrobial agents. Nonetheless, the number of cases with sepsis and the rate of mortality remain high (27). Recently, a monoclonal antibody to endotoxin, derived from human sources, has been shown to be partially protective in patients with septic shock (28). Another approach to the treatment of sepsis has been the administration of murine anti-TNF-α monoclonal antibodies (29). However, the use of murine antibodies in humans leads to the generation of anti-murine antibodies (29), which could hamper the action of the anti-TNF-α antibodies during repeated or chronic administration.

The observation that TNFR-IgG provides protection against endotoxin-induced lethality when given before and shortly after endotoxin administration suggests that this molecule may offer clinical potential both prophylactically in patients at high risk of sepsis and therapeutically in patients with shock. In contrast to murine anti-TNF-α antibodies, TNFR-IgG is derived from human proteins and therefore is expected to be much less immunogenic in humans, as indeed is the case for the similarly constructed CD4-IgG (A.A. and D.J.C., unpublished results). In addition, the increased affinity of TNFR-IgG for TNF-α appears to confer greater efficiency in blocking TNF-α *in vitro*, as compared with sTNFR or anti-TNF-α antibodies, although this remains to be investigated *in vivo*. Finally, since the rise in circulating TNF-α is subsequent to the occurrence of endotoxemia, it may be possible to extend the time window for treatment of septic shock by combination therapy with anti-endotoxin antibodies and anti-TNF-α agents such as TNFR-IgG.

We thank David Peers for help in TNFR-IgG purification, Greg Bennet for anti-TNFR antibodies, Kathy Kosewic and Laura Closkey for assay support, Dr. Chris Clark for comments on the manuscript, and Carol Morita for graphics.

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